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Recovery of Cholesterol and Triacylglycerol in Very-Fast Ultracentrifugation of Human Lipoproteins in a Large Range of Concentrations

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Summary: Very-fast ultracentrifugation using a benchtop ultracentrifuge was applied to the analysis of lipoproteins in 0.5 ml of human plasma. VLDL, IDL and LDL were flotated at densities of 1.006, 1.019 and 1.063 kg/l in runs lasting 30, 100 and 100 minutes. Chylomicrons, if present, were flotated in a separate run. HDL were isolated by precipitation of the apolipoprotein B-containing lipoproteins from total plasma using polyethylene glycol. Three series of separations were routinely performed: 1. VLDL run alone (632 samples), 2. VLDL run + LDL run (122 samples), and 3. Chylomicron separation + VLDL run + IDL run (92 samples). The concentrations of cholesterol and triacylglycerol were obtained for plasma, chylomicrons, VLDL, IDL, LDL and HDL. Plasma values ranged from 1.8 to 37.1 mmol/l cholesterol and 0.26 to 50.2 mmol/l triacylglycerol. The plasma triacylglycerol concentrations were corrected for free glycerol by 3% (for triacylglycerols < 2.5 mmol/l) and by 2% (for triacylglycerols $\geqq 2.5$ mmol/l). The recovery rate of lipids after ultracentrifugation was determined by comparing the concentrations in lipoproteins and in plasma. It was near to 100% and decreased for samples with extremely high lipid concentrations.

Introduction

Separation of lipoproteins of human plasma by ultracentrifugation can now be accomplished in a short time and starting with small samples, by use of vertical rotors (1), a benchtop ultracentrifuge (2–7), or density gradients (1, 6, 8). In "very-fast ultracentrifugation" (9) using a benchtop ultracentrifuge at 120 000 min^{-1}, the centrifugal field reaches 625 000 g which is about three times more than in classical ultracentrifugation. There are only minimal differences in the apparent lipid concentrations of lipoproteins separated by very-fast ultracentrifugation in comparison to conventional ultracentrifugation at 30 000 min^{-1}. As an advantage, VLDL, IDL and LDL[1]) are obtained albumin-free. The literature data cited were obtained on small numbers of samples, or within narrow limits of lipid concentrations. The aim of the present paper was therefore to study the recovery of cholesterol and triacylglycerol in separated lipoproteins in a large range of concentrations. 819 plasma samples were processed by very-fast ultracentrifugation.

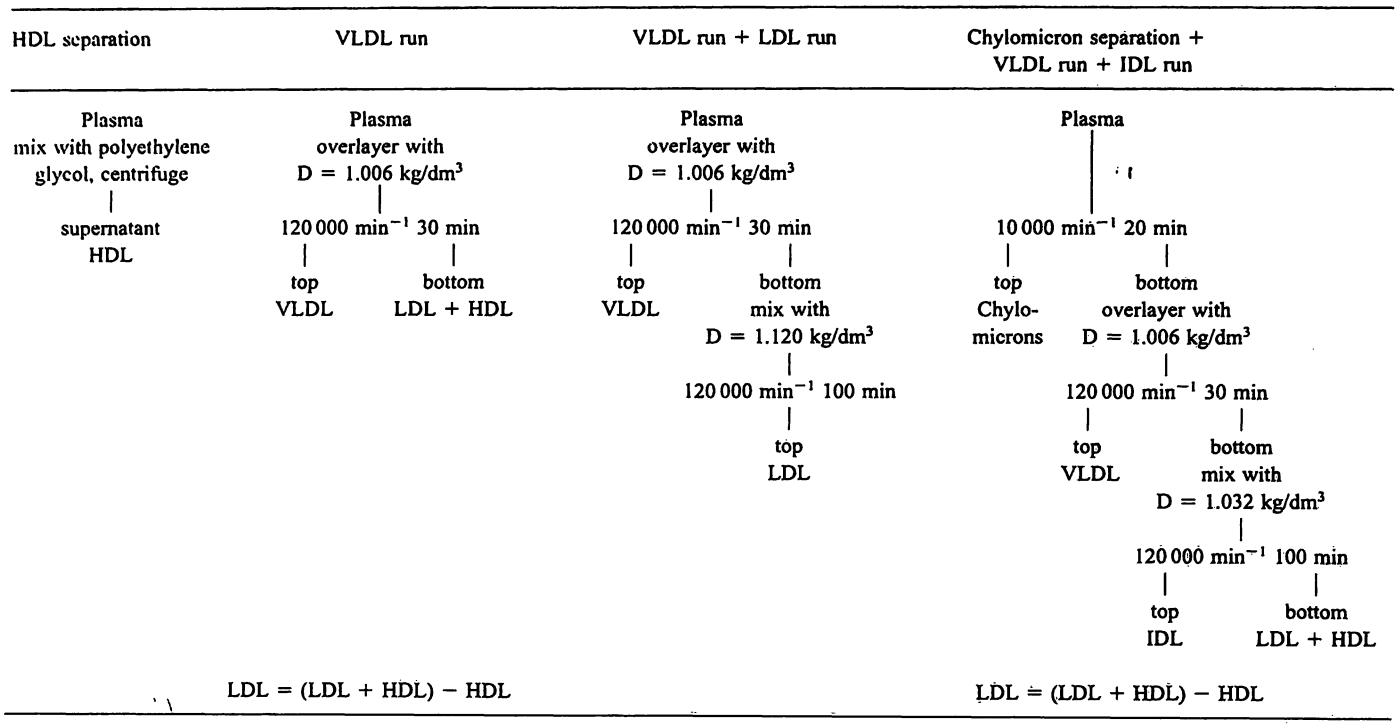
[1]) Abbrevations: VLDL = very-low density lipoproteins,
LDL = low-density lipoproteins,
IDL = intermediate-density lipoproteins,
HDL = high-density lipoproteins,
s_f = flotation (sedimentation) constant in *Svedberg* units (10^{-13} seconds).

Materials and Methods

Plasma samples and separation strategy (tab. 1)

Blood samples were taken by venipuncture after an overnight fast and collected into evacuated tubes (Red monovette, Sarstedt, Nümbrecht-Rommelsdorf, Germany) containing 1.2–2 g of K_2-EDTA per litre as an anticoagulant and antioxidant. Plasma was obtained by centrifugation (6 minutes at 2500 g) at room temperature. Chylomicrons were separated from the fresh plasma if necessary. The material was stored for maximally five days at 4 to 8 °C before ultracentrifugation. Three types of separation were routinely performed:

Tab. 1 Flow sheet of separation procedures



1. VLDL run alone (632 samples with 1.8−15.8 mmol/l cholesterol and 0.26−46.4 mmol/l triacylglycerol)

2. VLDL run followed by LDL run (122 samples with 2.6−18.2 mmol/l cholesterol and 0.59−13.0 mmol/l triacylglycerol)

3. Chylomicron separation followed by VLDL run and by IDL run (92 samples with 3.4−37.1 mmol/l cholesterol and 1.8−50.2 mmol/l triacylglycerol). In all samples, HDL were isolated from total plasma by precipitation using polyethylene glycol.

Separation of chylomicrons

Chylomicrons were separated if one of the following criteria were fulfilled:

1. Triacylglycerol concentration above 7 mmol/l

2. Lipaemic plasma or

3. History of chylomicronaemia.

Plasma (5 ml) was centrifuged at 20 °C and 19 000 min^{-1} (centrifugal field = 11 000 *g*) for 20 minutes including 4 minutes for acceleration and deceleration. The conditions were adequate to complete flotation of $s_f = 400$ particles. The chylomicron-free plasma was carefully aspirated using a syringe with wide needle. The further ultracentrifugation programme included separation of IDL (see below).

Very-fast ultracentrifugation

We used the Optima™ TLX Ultracentrifuge with rotor TLA-120.2 (r_{max} = 38.9 mm, r_{min} = 24.5 mm), thickwall polycarbonate tubes # 343778 (volume = 1 ml, for use without cap) and CentriTube Slicer (Beckman Instruments Inc). The runs were driven at 20 °C and at full speed of 120 000 min^{-1} which corresponds to a centrifugal field of 625 000 *g*. For isolation of VLDL, 0.500 ml of EDTA-plasma were overlayered with 0.500 ml of solution with density D = 1.006 kg/l. The mass of the tube content was determined by weighing. After centrifugation for 30 minutes, the tubes were sliced to yield about 0.550 ml of top and 0.450 ml of bottom fraction. Both fractions were carefully drawn off with a pipet. If necessary, recovery of the top fraction was completed by washing with additional volume of density medium. The accurate masses of both fractions were determined by weighing. For flotation of LDL at density D = 1.063 kg/l, 0.500 ml of the bottom fraction were mixed with 0.470 ml of solution with density D = 1.120 kg/l in a second centrifuge tube. The mass of the tube contents was measured. After centrifugation for 100 minutes, slicing and weighing were performed as before. Alternatively, for flotation of IDL at density D = 1.019 kg/l, 0.500 ml of the bottom fraction of VLDL run were mixed with 0.470 ml of solution with density D = 1.032 kg/l. After slicing as described above, the bottom fraction was analyzed for LDL + HDL. The concentrations of cholesterol and triacylglycerol were determined in the top and bottom fractions. From the apparent concentrations ('), the lipid concentrations in the lipoproteins were calculated considering the type of separation:

$$VLDL = VLDL' \cdot (2 \cdot m_2/m_1)$$

VLDL run exclusively:

$$(LDL + HDL) = (LDL + HDL)' \cdot (2 \cdot m_3/m_1)$$

VLDL run + LDL run:

$$LDL = LDL' \cdot (2 \cdot m_3/m_1) \cdot (2 \cdot m_5/m_4)$$

Chylomicron run + VLDL run + IDL run:

$$IDL = IDL' \cdot (2 \cdot m_3/m_1) \cdot (2 \cdot m_5/m_4)$$
$$(LDL + HDL) = (LDL + HDL)' \cdot (2 \cdot m_3/m_1) \cdot (2 \cdot m_6/m_4)$$

m_1: total mass in first (VLDL) run,
m_2: top (inclusive wash solution) mass,
m_3: bottom mass after first run,
m_4: total mass in second (IDL or LDL, resp.) run,
m_5: top mass,
m_6: bottom mass after second run.

In fact, the volume ratio v_a/v_b (v_a = volume after slicing, v_b = volume before ultracentrifugation = 0.5 ml) of the analyte was considered; for instance, $VLDL/VLDL' = v_a/v_b = m_2/(m_1 \cdot 0.5) = 2 \cdot m_2/m_1$. Typical values were: m_1 = 1020 mg, m_2 = 450 mg, $VLDL/VLDL' = 0.8824$. The density media were prepared as follows:

Solution 1. D = 1.006 kg/l, pH = 9.18: 11.4 g NaCl + 0.1 g EDTA-Na + H_2O ad 1 l + 1 ml NaOH 1 mol/l.

Solution 2. D = 1.182 kg/l, pH = 7.32: 124.9 g NaBr + solution 1. ad 500 ml.

Solution 3. D = 1.032 kg/l, pH = 8.26: 50 ml solution 1. + 8.7 ml solution 2.

Solution 4. D = 1.120 kg/l, pH = 7.38: 50 ml solution 1. + 92 ml solution 2.

The densities were determined using the oscillator device DMA 602 (Anton Paar AG, Graz, Austria).

Other analyses

HDL were isolated from total plasma using a 95 g/l polyethylene glycol 20000 solution in phosphate buffer, pH = 6.5 (10). This reagent precipitates apolipoprotein B containing lipoproteins and also yields quantitative separation in lipaemic samples. When no LDL run was performed, lipid concentrations in LDL were calculated as the difference of concentrations of the infranatant of either VLDL or IDL run (= LDL + HDL) and HDL (11). The concentrations of cholesterol and triacylglycerol in whole plasma were determined by enzymatic reactions (CHOD-PAP method, GPO-PAP method) using commercial test kits (Boehringer Mannheim) on a 550 Express Analyzer (Ciba Corning). Analyses on lipoprotein solutions after ultracentrifugation were done in the same way. Measurements of low concentrations (triacylglycerol in LDL and in HDL, cholesterol in VLDL and in HDL) were performed with tenfold increased sample to reagent ratio. Calibration and control samples were in this case applied at the same ratio. Samples with very high lipid concentrations were diluted. The day-to day imprecision of lipid determinations was on the order of 1.5% for cholesterol and 2.5% for triacylglycerol.

Glycerol correction

The triacylglycerol test kit provided the sum of acyl glycerides and free glycerol. Correction was necessary to make the triacylglycerol in plasma and in lipoproteins comparable. Therefore, the concentration of free glycerol was tested in 63 randomly selected plasma samples by the UV test initiated with glycerokinase. Before the test, the plasma was cleared by precipitation with trichloroacetic acid: The test combination triacylglycerol (Boehringer Mannheim) was used. We found a significant relationship (correlation coefficient $r = 0.273$, $p < 0.030$) between the concentrations of free glycerol and triacylglycerol. Based on this, the plasma triacylglycerol was approximately corrected by 2% for concentrations $> = 2.5$ mmol/l and by 3% for concentrations < 2.5 mmol/l.

Results

Lipoprotein lipid concentrations were calculated as the sum of the lipid concentrations in the lipoproteins. They were compared to the plasma lipid concentrations. There were good correlations in the three types of analyses performed: for VLDL runs alone (figs. 1 and 2), for the combination of VLDL and LDL runs (fig. 3) and for the combination of chylomicron, VLDL and IDL runs (figs. 4 and 5). As outlined in the methods section, analysis of HDL by precipitation influenced the sum of lipoprotein lipids only in the combination of VLDL and LDL runs, while in the other types of analyses the sum was calculated using (LDL + HDL). The recovery of cholesterol and triacylglycerol is summarized in table 2. The bias (= 100 − recovery; lower part of the figures) was generally symmetrical to zero in low and medium lipid concentrations but tended to become negative for cholesterol and triacylglycerol concentrations above 7 mmol/l. The bias was calculated immediately after every analysis. When it exceeded ± 20%, the analysis was repeated. This was necessary in 18 (2.8%) VLDL runs, in 3 (2.5%) VLDL plus LDL runs, and in 6 (6.5%) chylomicron plus VLDL plus IDL runs.

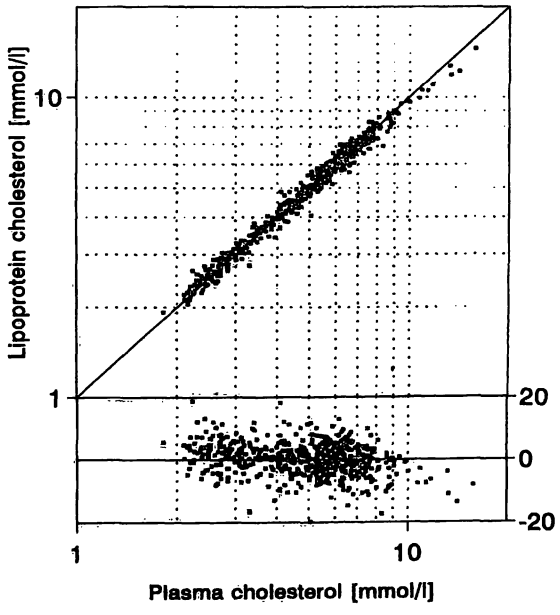


Fig. 1 Recovery of cholesterol (logarithmic scale) after VLDL run (n = 632).
Lower part: bias (%) = (lipoprotein cholesterol/plasma cholesterol) · 100 − 100.

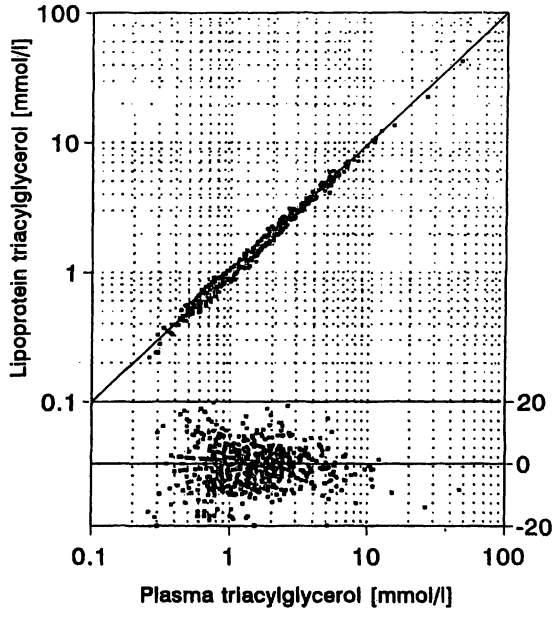


Fig. 2 Recovery of triacylglycerol (logarithmic scale) after VLDL run (n = 614).
Lower part: bias (%) = (lipoprotein triacylglycerol/plasma triacylglycerol) · 100 − 100.

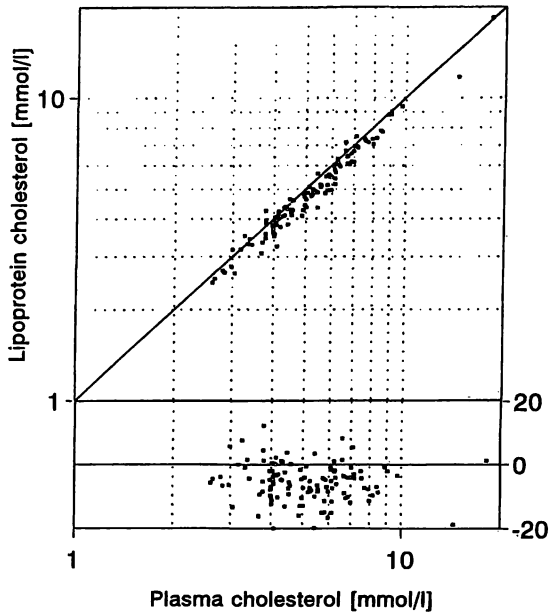


Fig. 3 Recovery of cholesterol (logarithmic scale) after VLDL plus LDL runs (n = 119).
Lower part: bias (%) = (lipoprotein cholesterol/plasma cholesterol) · 100 − 100.

Discussion

It can be estimated by calculation that the short run periods we chose were sufficient to achieve the separations at the midpoint of the tubes. Concerning the VLDL run, 30 minutes were enough for all light lipoproteins to flotate into the upper half of the tubes. LDL was not expected to sediment since the plasma was overlayered with the medium. In contrast, before the IDL and LDL runs the density media were added by mixing. The separation quality was in these cases critically dependent on sedimentation of the heavier lipoproteins. The run time of 100 minutes corresponded to $s_f = 2.1$ for flotation and $s_f = 2.8$ for sedimentation at the midpoint of the tube, i. e., it was sufficient for the expected lipoprotein separations. The recovery of lipoprotein lipids approached 100% in low and medium lipid concentrations while it became lower in extremely high lipid concentrations.

Comparable data on the recovery rate were only given by one other group (6). The good recovery was based on the slicing technique combined with washing steps. The calculation of the lipoprotein lipid concentrations

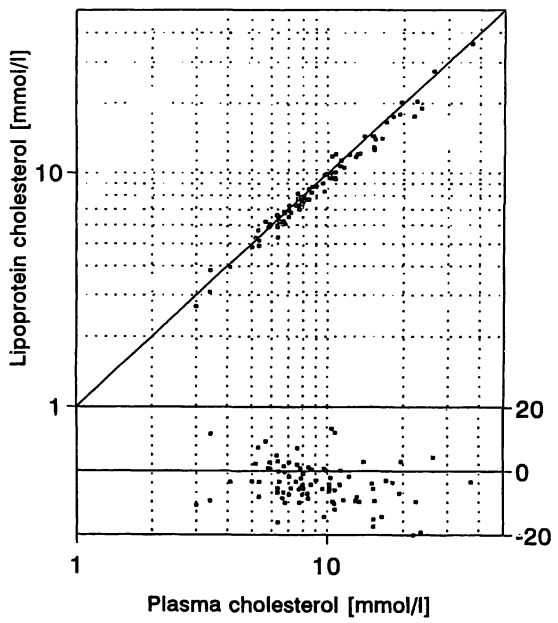


Fig. 4 Recovery of cholesterol (logarithmic scale) after chylomicron plus VLDL plus IDL runs (n = 86).
Lower part: bias (%) = (lipoprotein cholesterol/plasma cholesterol) · 100 − 100.

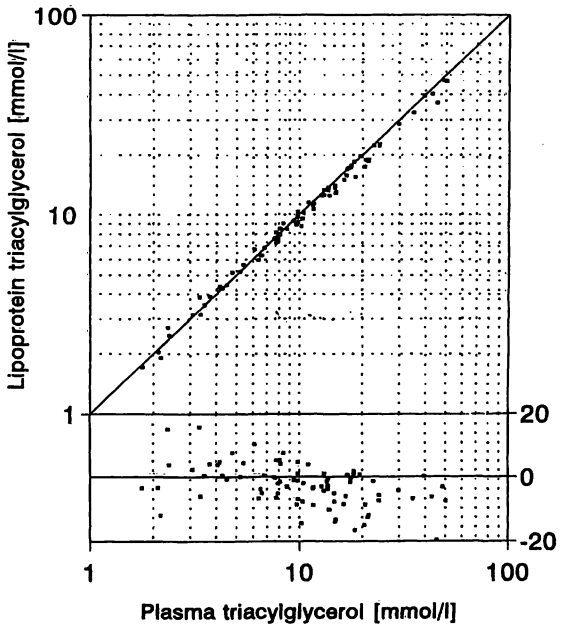


Fig. 5 Recovery of triacylglycerol (logarithmic scale) after chylomicron plus VLDL plus IDL runs (n = 82).
Lower part: bias (%) = (lipoprotein triacylglycerol/plasma triacylglycerol) · 100 − 100.

Tab. 2 Mean recovery (%) of cholesterol and triacylglycerols

	Triacylglycerol		Cholesterol	
	Mean	95% tolerance interval	Mean	95% tolerance interval
VLDL run	100.1	91.5 − 108.6	99.2	87.4 − 111.0
VLDL + LDL run	94.4	83.5 − 105.4	n.d.	
Chylomicron + VLDL + IDL run	96.0	83.7 − 108.4	97.4	85.1 − 109.6

n. d. = not detected

from the apparent concentrations was based on weighing which was much more accurate and time-saving than volume measurements. In the case of triacylglycerol, the recovery was dependent on the glycerol correction. We found the recommended correction by 0.12 mmol/l inadequate, namely in the case of very low triacylglycerol concentrations. Instead of this, we used corrections of 3% below and 2% above the level of 2.5 mmol/l triacylglycerol.

The proposed method of very-fast ultracentrifugation has the advantage of being fast. It yields nearly complete recovery of lipoproteins. It requires only 0.5 ml of plasma sample which can have lipid concentrations ranging from extremely low to extremely high.

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